TABLE I. SUBSTRATE UTILIZATION

| $^{14}C$ label | Other substrate (1 mg/ml) | Oxygen utilization (µl/100 mg wet wt/hr) | Total lactate production (µl/100 mg wet wt/hr) | | Labeled substrate as precursor: $CO_2$ production (µmol x $10^{-3}$/100 mg wet wt/hr) | | Lipid production (µmol x $10^{-3}$/100 mg wet wt/hr) | | Protein production (µmol x $10^{-3}$/100 mg wet wt/hr) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic | Aerobic | Anaerobic |
| Glucose | None | 26±0.7 (107) | 82± 6 (12) | 62± 4 (12) | 28± 1 (12) | 0 (12) | 2±0.2 (12) | 0.6±0.1 (12) | 4±0.2 (12) | 0 (12) |
| | Lactate | 22±2 (6) | 80±11 (24) | 80±12 (20) | 27± 0.7 (5) | 6±2 (5) | 3±0.4 (6) | 0 (6) | 4±0.6 (6) | 0 (6) |
| | Acetate | 35±1 (12) | 70± 0.3 (6) | 76± 7 (5) | 32± 4 (5) | 4±1 (5) | 4±0.3 (6) | 1±0.2 (5) | 2±0.3 (6) | 0 (6) |
| Lactate | None | 35±3 (6) | 6±17 (6) | | 201±14 (6) | 0 | 41±6 (6) | 0 (6) | 19±2 (6) | 0 (6) |
| | Glucose | 29±3 (6) | 94±21 (6) | 94±12 (6) | 124±26 (4) | 28±4 (6) | 5±1 (5) | 0 (6) | 18±2 (5) | 0 (6) |
| Acetate | None | 22±2 (6) | 3± 0.8 (6) | 6± 3 (5) | 73±14 (5) | 8±6 (5) | 2±0.5 (6) | 0 (6) | 9±3 (6) | 0 (6) |
| | Glucose | 36±3 (5) | 76± 4 (5) | 85± 7 (6) | 61± 8 (5) | 6±1 (3) | 3±0.3 (6) | 0 (6) | 6±2 (6) | 0 (6) |

Values are mean ± standard error of mean

1003540161



Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 4 1974

Application for Research Grant
(Use extra pages as needed)

Date: January 24, 1974

1. Principal Investigator (give title and degrees): Herbert W. Wallace, M.D.
Associate Professor of Surgery

2. Institution & address:
Graduate Hospital of the University of Pennsylvania
19th and Lombard Streets
Philadelphia, Pennsylvania 19146

3. Department(s) where research will be done or collaboration provided:
Department of Surgery, Harrison Department of Surgical Research,
Department of Physiology

4. Short title of study: PULMONARY BIOCHEMISTRY

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: Five years

7. Brief description of specific research aims:

1. To determine the normal metabolic pathways and the primary substrates utilized as energy sources by pulmonary tissue;
2. To determine the regulatory mechanisms of these metabolic pathways;
3. To study the interrelationships between the metabolism of substrate and the synthesis of byproducts such as phospholipids and possibly prostaglandins;
4. To evaluate the effects of tobacco smoke upon the metabolism of the pulmonary parenchyma;
5. To compare the metabolic processes of animal experiments with those of normal human pulmonary tissue.

1003540155

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

give rise to different parts of the phospholipids made by the lung. Synthesis of phospholipids and surfactants appear to be important biosynthetic functions of the lung (13). The results of Felts (14) suggest the possibility that the $^{14}C$ from glucose tends to go predominantly into the glycerol fragment. This suggested compartmentalization between the carbohydrate and lipid utilization pathways might involve some very interesting regulatory aspects. Without "hard" numerical data, it is pointless to speculate on the possibilities.

Double labeling experiments under aerobic and anaerobic conditions will be performed in which both glucose and acetate or fatty acid are labeled, the former with $^{14}C$ and the latter with tritium. Following quenching in the usual manner with perchloric acid and neutralization, the lipids will be extracted with acetone:ether:chloroform (2:2:1), and the lipid fraction will be counted for $^{14}C$ and $^{3}H$.

Hydrolysis in boiling 1M NaOh should lead to hydrolysis of the lipid into glycerol and fatty acid and inorganic phosphate (from phospholipid). Ether extraction under alkaline conditions will extract the glycerol and leave the fatty acid in the alkaline solution. Counting the glycerol for $^{3}H$ and $^{14}C$ radioactivity should indicate its origin. The alkaline solution is then acidified, rendering the fatty acids insoluble, and they can be extracted with chloroform and then counted for the double radioactivity. The residual acidified aqueous solution should be counted also as a check for incomplete extractions. Analysis of the distribution of label will indicate the origin of the various components of the lipid fraction.

6. Determination of the effects of $CO_2$ and $O_2$ on substrate utilization and biosynthesis.

Two groups of experiments are contemplated. The first deals with the utilization of approximately 5.6% $CO_2$ in the atmosphere during incubation, since this is the approximate concentration of $CO_2$ in the alveolar gas. It is of interest to investigate whether this elevated $CO_2$ is of any biosynthetic importance. In the second group of experiments $PO_2$ will be regulated, utilizing concentrations such as 100%, 70%, 50%, and 30% oxygen in order to determine if the $PO_2$ has any regulatory effect on the substrate utilization.

7. Evaluation of isolated mitochondrial regulation.

Because of the regulatory importance of the Krebs cycle enzymes and intermediates, we plan early investigations in this area, which will aid in the interpretation of the data of other experiments. The special enzymatic mechanisms by which the Krebs cycle intermediates can be regulated and replenished are called anaperotic reactions. Recent research has shown that the pyruvate dehydrogenase system is also inhibited by ATP, which is therefore a negative modulator. Whenever the ATP level in the cell exceeds a certain point, the pyruvate dehydrogenase system, which provides fuel for the tricarboxylic acid cycle, "turns off." In addition, it has been shown that the NAD-linked isocitrate dehydrogenase is the normal catalyst for isocitrate oxidation in the tricarboxylic cycle; the NADP-linked enzyme is primarily concerned with auxiliary biosynthetic reactions of the cycle (3,16).

1003540165

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The research facilities of the Department of Surgery and the Department of Physiology of the University of Pennsylvania School of Medicine will be available for these studies. (The principal investigator has a joint appointment in these departments.) There are animal laboratory facilities at both the Graduate Hospital and the Harrison Department of Surgical Research of the University of Pennsylvania. The major pieces of equipment needed for radioisotopic determinations and biochemical determinations are available to us. If needed in the future, larger animals and animals of other species are readily obtainable through the facilities of the School of Veterinary Medicine of the University of Pennsylvania. In addition, Dr. Leena Mela, a disciple of Dr. Britton Chance, has made her laboratory facilities available for collaborative studies.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Herbert W. Wallace, M.D.
Leena M. Mela, M.D.
T. Peter Stein, Ph.D.
Ellen M. Liquori, M.S.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
Attached.

10035401G9

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

of the tissue that was analyzed, nor was there any detectable loss of $^{14}C$-lactate from the incubation solution. The total lactate of the incubation medium increased during these experiments.

These preliminary experiments clearly indicate that rat lung slices are incapable of utilizing lactate as a substrate. In fact, the amount of lactate produced by the lung in the presence of excess lactate was unchanged.

In another group of experiments, $^{14}C$-labeled octanoic acid bound to albumin (by the method of Millstein and Driscoll; 29) was employed as a metabolic substrate in the presence and absence of glucose anaerobically. No significant uptake of the fatty acid was detected, nor was radioactivity found in the collected carbon dioxide, indicating that this fatty acid is not a major energy source under these experimental conditions. Further experiments with palmitate are contemplated.

These data seem to indicate that the metabolic processes of pulmonary tissue are primarily an anaerobic process. The use of lung slices may be criticized, and our data are preliminary and incomplete and will require additional experiments to develop significant statistical data. However, they are supported by the recent studies of lung mitochondria by Fisher et al. (personal communication; 22) which suggest that little oxidative phosphorylation occurs. They feel that the moderate amount of ATP produced is utilized during phospholipid synthesis under the control of calcium ion.

b. Biochemistry of the tracheobronchial mucosa.

Many of the investigations of the effects of smoking on the tracheobronchial tree have been studies of ciliary action. Although the tracheobronchial mucosa has been pointed out to be one of the prime targets of the effects of smoking, in a careful review of the literature we failed to uncover any definitive biochemical studies of the mucosa of the tracheobronchial tree.

In an effort to obtain baseline values, we have performed preliminary studies on bovine tracheal and bronchial mucosa using standard Warburg techniques. In 85 experiments the average rate of oxygen uptake of cow tracheal mucosa was 30 ± S.D. 8 µl $O_2$ per hour per 100 mg wet weight, and the bronchi showed a similar uptake. A piece of human trachea obtained during a medically-indicated tracheostomy under anesthesia revealed an oxygen uptake of 16 µl $O_2$ per hour per 100 mg wet weight. Two samples of human main bronchial mucosa obtained from fresh surgical specimens gave results of 19 µl $O_2$ per hour per 100 mg wet weight. A piece of human lung was 14 µl $O_2$ per hour per 100 mg wet weight. Utilizing $^{14}C$-1-glucose, we also measured glucose consumption, $CO_2$ production and incorporation of radioactivity into lipids, protein and RNA. In six experiments glucose uptake averaged 4.9 µg per 100 mg wet weight per hour, with 41.5% of the metabolized glucose accounted for by $CO_2$, 29.6% in inorganic lipids, 7.6% in RNA, and 21.3% in protein. Two experiments with human bronchial mucosa revealed a glucose uptake of 3 µg per 100 mg of wet tissue per hour, with 47% accounted for by $CO_2$, 32% in protein, 18% in RNA, and 3% in inorganic lipids. Two pieces of normal human lung had an average glucose uptake of 40.5 µg per 100 mg wet weight per hour, with 67.8%

10035401 60

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

16. Other sources of financial support: NONE
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| | | | |

PENDING OR PLANNED NONE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| | | | |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Herbert W. Wallace

Signature [signature] Date 1/25/74

Telephone 215 (Area Code) KI 6-5151 (Number) -- (Extension)

Checks payable to

Mailing address for checks

Responsible officer of institution

Typed Name

Title

Signature Date

Telephone (Area Code) (Number) (Extension)

10035401Z4

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

In a series of experiments, mitochondria of rat or rabbit lungs will be harvested by techniques of differential centrifugation. Oxygen uptake with various substrates, particularly fatty acids as substrates (palmitate) will be measured with a polargraph. Utilization of these substrates will be determined, and the effects of metabolic inhibitors will be studied. Either glycolysis or fatty acid oxidation may be responsible for providing the CoA for these mitochondria. The fact that lactate is noted to increase in quantity would indicate the probability that pyruvate is not the prime source of CoA. The energy produced can be utilized either for ATP production or for ion transport. Measurements of ATP, ATPase and ion transport (particularly Ca++) will be performed utilizing the procedures outlined by Mela and Chance (16). If palmitate appears to be an important substrate, the possible requirement of carnitine will be investigated. We will compare the in vitro results with those obtained in the study of other tissues, including liver, and their mitochondria. This information will enable us to characterize oxygen and substrate characteristics of the lung. The basic techniques have been well utilized in the study of liver by J. R. Williamson of this institution. We plan to use his techniques of fluorometric assay, which are described in his numerous papers and summarized in Methods of Enzymology (4). From his experiments Dr. Williamson was able to define certain regulatory mechanisms involved in liver synthesis and metabolism, and the utilization of these techniques should lead to very useful information regarding pulmonary metabolism. These techniques will allow for the evaluation of Acetyl CoA as well as pyridine nucleotide oxidation-reduction ratios, which will enable us to evaluate the total energy potential of the system. Such information should enable us to procede to a more detailed investigation of individual reactions.

8. Isolated, perfused lung preparation.

Perfused whole lungs appear to be superior to tissue slices for the study of lung metabolism (17). Lungs will be removed from fasted rabbits, ventilated by a piston pump with gas containing 5% $CO_2$ in air, and perfused in a pulsatile manner via the pulmonary artery and veins with Krebs bicarbonate buffer solution containing 6% serum albumin. A closed system with a venous reservoir will be used for the perfusion. The lungs will be weighed, and the total volume of the perfusate will be measured by dilution of Evans Blue. The preparation will be immersed in Krebs solution equilibrated with $CO_2$ in air. Various substrates will be investigated, including glucose, lactate, amino acids, and selected fatty acids. Aliquots of the perfusion mixture will be taken at 30-minute intervals and analyzed for glucose, lactate, and pyruvate. Biopsies of the lung will be taken by rapid freezing between tongs precooled in liquid $N_2$, and the tissue will be analyzed by fluorometric methods (4) for pyruvate/lactate, α-glycerophosphate/dihydroxyacetone phosphate, β-OH butyrate/acetoacetate and NADH/NAD levels to determine redox state (18) and ATP will be measured to evaluate energy supply. Similar experiments will be performed with the addition of lactate instead of glucose to the perfusate. In some experiments, $^{14}C$-1 or $^{14}C$-6 labeled glucose will be added to the perfusion medium; $^{14}CO_2$ will be recovered by passing the expired air through a $CO_2$ absorber, and the trapped radioactivity will be measured by scintillation counting. The ratio between $CO_2$ produced from $C_1$ and $C_6$ labeled glucose will be used as an index of hexosemonophosphate shunt activity.

1003540166

The significance of the α-glycerophosphate pathway as an NADH shuttle mechanism between cytoplasmic and mitochondrial spaces in lung cells will be studied by experimentally manipulating the tissue level of α-glycerophosphate, as described in section 2.

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

vasoactive amines, such as angiotensin II, adrenalin, oxytocin, vasopressin and histamine were not affected by passage through the pulmonary circulation. In both in vivo and in vitro animal studies he has shown that anaphylaxis, shock, and even ventilation cause the release of prostaglandin from the lung, which strongly indicates a biosynthetic process. The relationship of these metabolic processes to blood gas transport and pH would be interesting and important to elucidate. Recently, Holub (9), utilizing alveolar cells obtained from washings of rabbit lungs, demonstrated the possibility that these cells are involved in antibody formation. Some of these cells have also been shown to have phagocytic properties, which seem to be related to catalase-dependent peroxidative metabolism (10).

Much of the recent investigation has been focused on cells derived from lavage of the lung, particularly the macrophage. It has been suggested that the macrophage has a controlling role in carcinogenesis (11). The exact origin of the alveolar macrophages remains controversial. Some investigators, including Sorokin (12), believe that they originate primarily from lung tissue, while others, such as Pinkett et al. (13), have presented evidence in support of a peripheral hemopoietic tissue origin. Studies done by Massaro et al. (14) indicate that the alveolar macrophage is capable of synthesizing phospholipids and that glucose seems to be a source of energy for this process.

We originally became interested in these problems after reading a paper by Weber and Visscher (15), whose experiments showed that isolated, perfused lung produced a significant amount of lactic acid, even when the perfusate had a concentration of lactic acid greater than 100 mg/100 ml. These studies were done aerobically with excellent control of the perfusate, etc., and it was difficult to understand why an organ with the greatest access to oxygen of any in the body would need to produce lactic acid under aerobic conditions. Glaviano et al. (16) reported a significant pulmonary A-V difference of lactate, which appeared to increase with sympathetic stimulation. In 1958 Bucheri et al. (17) studied the A-V difference of lactic acid in 27 patients during cardiac catheterization and found a significant increase in lactic acid in the left atrial blood as compared to the pulmonary artery blood. Lochner and Nasseri (18) in studies of perfused lung demonstrated a lactic acid production of 4.78 mg/min per gm wet weight of tissue. In 1966 Salisbury-Murphy (19) utilized $^{14}C$-labeled glucose at both the C-1 and C-6 positions and noted a $CO_2$ ratio for $^{14}C\text{-}1/^{14}C\text{-}6$ of 2.5 for lung slices. In 1967 Wolfe et al. (20), utilizing isolated pneumocytes, found a $C^{-1}/C^{-6}$ ratio of 7. Both of these studies indicate a large hexose monophosphate shunt. Histochemical studies by Tyler and Pearse (21) revealed an absence of succinic dehydrogenase in alveolar cells. However, in the more recent work of Wolfe et al. (20) this enzyme was thought to be present. Felts (2) used evenly labeled $^{14}C$-glucose with lung slices and observed that little $^{14}CO_2$ was produced, nor was much radioactivity found in the total fatty acids. The radioactivity that could be detected was primarily in the phospholipid fraction. In recent unpublished experiments my colleague Dr. Aron Fisher ventilated dogs using a tracheal divider. One lung was respired with room air and the other with 100% nitrogen for a 3-4 hour period. Subsequent electron microscopic studies of the lungs showed no alterations or differences between normoxic and hypoxic pulmonary tissue.

10035401S7

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

14. First year budget:

A. Salaries (give names or state "to be recruited")

| Professional (give % time of investigator(s) even if no salary requested) | | % time | Amount |
|---|---|---|---|
| Herbert W. Wallace, M.D. | Principal Investigator | 40% | $18,075 |
| T. Peter Stein, Ph.D. | Biochemist | 50% | 9,038 |
| Leena M. Mela, M.D. | Consultant | 5% | 0 |
| **Technical** | | | |
| Ellen M. Liquori, M.S. | Research Technician | 100% | 12,075 |

Sub-Total for A 39,188

B. Consumable supplies (by major categories)

| | |
|---|---|
| Rats | $1,200 |
| Rat care @ $0.04/day | 200 |
| Chemicals, glassware, tubing | 2,500 |
| Radioisotopes | 2,000 |

Sub-Total for B 5,900

C. Other expenses (itemize)

| | |
|---|---|
| Travel (domestic) | 600 |
| Publications | 400 |

Sub-Total for C 1,000

Running Total of A + B + C 46,088

D. Permanent equipment (itemize)

| | |
|---|---|
| Fluorometer | 6,000 |
| Recorder | 1,200 |

Justification: A fluorometer and a recorder are not available to us and will have to be purchased and modified for the purposes described in this proposal.

Sub-Total for D 7,200

E. 6,913

Total request $ 60,201

E. Indirect costs (15% of A+B+C) * 5% annual increase

15. Estimated future requirements: **Upkeep & repair of equipment

***Includes cost of buying, boarding and operating on dogs as well as rats

| | Salaries * | Consumable Suppl. | ***Other Expenses | Permanent Equip. ** | Indirect Costs | Total |
|---|---|---|---|---|---|---|
| Year 2 | 41,147 | 7,500 | 1,000 | 500 | 7,447 | 57,594 |
| Year 3 | 43,204 | 7,500 | 1,000 | 500 | 7,756 | 59,960 |

10035040120

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

8. Brief statement of working hypothesis:

Smoking has been accused of being a major causative factor in pulmonary cancer as well as a serious aggravating factor in other pulmonary diseases. However, most of the evidence for and against these accusations has been inconclusive and in many instances without good scientific basis. The purpose of this investigation is to elucidate the normal metabolic processes of pulmonary tissue and their regulation. Without these data, it is impossible to evaluate the metabolism or transport of other agents in pulmonary tissue and their relationship to metabolic alterations and neoplastic pulmonary disease. After completing baseline studies of pulmonary metabolism, we propose to evaluate the effects of tobacco smoke and/or its various components and attempt to elucidate these effects at a cellular level.

9. Details of experimental design and procedures (append extra pages as necessary)

A. Background.

1. Work accomplished by other investigators.

At the 1970 meeting concerning the morphology of experimental respiratory carcinogenesis sponsored by the NCI and the AEC, it was pointed out that a thorough understanding of the structural and functional components of the respiratory tract is necessary before and meaningful exploration of the causes of disease in this complex organ system can be undertaken. Biochemical and histochemical studies indicate that all major routes of carbohydrate metabolism appear to operate in mammalian lungs. These include the presence of the Embden-Meyerhof glycolytic pathway, the citric acid cycle, and the hexose monophosphate shunt. Several investigators, including Felts (1,2) have studied the utilization of lipids and their derivatives for synthetic purposes, but there is little clearcut evidence and much controversy concerning the role of lipids as an energy source for the lung. Studies on rats have indicated that lung slices can oxidize fatty acids (2,3). However, mitochondrial preparations of rabbit lungs reportedly have limited ability to oxidize β-hydroxybutyrate or other fatty acids (4). Although it is controversial whether lipid substrates are an important energy source for the lung, pulmonary tissues of all animals have been found to incorporate fatty acids for lipid synthesis (5,6). Oxygen consumption in the lung appears to be principally by the cytochrome chain, but there is some evidence that a substantial amount is used by enzyme systems that are not inhibited in the presence of cyanide in the incubation medium (7). However, it must be pointed out that under most circumstances of tissue incubation obtaining a complete block of oxidative phosphorylation by any of the known inhibitors can be difficult. Sorokin (8) has pointed out that pulmonary development can occur in vitro despite the presence of a high cyanide concentration in the medium; thus a noncytochrome-mediated oxygen metabolism might be involved in bioxynthetic reactions. Dr. J. R. Vane (personal communication) of the Royal College of Surgeons points out that pulmonary tissue may have several functions besides that of transporting gases. He has shown that during perfusion of the lung with acetylcholine, bradykinin, 5-hydroxytryptamine and prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ were apparently inactivated by pulmonary tissue, whereas other

(CONTINUED ON PAGE 2a)

10035401156

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

results (Wallace and Stein, unpublished data).

Using standard Warburg techniques, we incubated rat lung slices in Krebs-Ringer's phosphate buffer at pH 7.4 with 0.1% glucose and observed lactic acid production under aerobic (room air) and anaerobic (100% nitrogen) conditions for 30, 60, 90, and 120 minutes. Oxygen uptake and lactic acid production were found to be linear. In a series of 18 experiments the average oxygen uptake under aerobic conditions was 19.2 µl/mg of nitrogen per hour. The lactic acid production of the tissue incubated with air was 37.5 µg/mg of nitrogen per hour, while that of the tissue incubated anaerobically was 41.3 µg/ml of nitrogen per hour, an insignificant difference.

In a series of pilot studies we repeated these experiments using cyanide as an electron transport inhibitor and again found that lactic acid production appeared equal under aerobic and anaerobic conditions. Because of the possibility that cyanide might interfere with the enzymatic determination of lactic acid through its interaction with pyridine nucleotides, we had attempted to remove the cyanide before conducting the analysis. Nevertheless, we felt that we would be on more secure ground if we used carbon monoxide. Therefore, we repeated these experiments under two conditions, using 1) 3% CO, 20% $O_2$ and 77% $N_2$; or 2) 100% CO, which was subsequently flushed out with the mixture of 3% CO, 20% $O_2$ and 77% $N_2$. The oxygen concentration was checked with a Beckman oxygen meter. Control experiments with zero time incubation, etc., were carried out. The average values for lactic acid production under aerobic and anaerobic conditions with nitrogen, air and carbon monoxide were similar. Glucose uptake in air averaged 144 µg/mg $N_2$/hr (15 experiments) and was similar with 100% CO (19 experiments).

These findings leave little doubt in our minds that lactic acid production is unaffected by the amount of oxygen available to the lung tissue. These preliminary data suggest that the major glucose pathway is not through the mitochondrial system. Another possibility is that the pathways of lactic acid production differ under aerobic and anaerobic conditions. Subsequent experiments will help to elucidate these problems.

In another experiment we used 0.1% glucose and 0.1% sodium acetate in the incubation medium. The sodium acetate was uniformly labeled with $^{14}C$. A slight increase in lactic acid production was observed under anaerobic conditions as compared to aerobic conditions. These reaction mixtures were fractionated by a method similar to that of Massaro et al. (14). It is interesting to note that under aerobic conditions the radioactivity from the acetate could be found in relatively large amounts in the protein fraction and to some extent in the lipid fraction, whereas anaerobically following an aerobic incubation, the initial radioactivity seen in the lipid fraction seemed to decrease, possibly due to utilization of lipids as an energy source. Some of the radioactive $CO_2$ noted under these anaerobic conditions may have been due to decarboxylation. In an additional series of experiments, $^{14}C$-labeled lactate was added as a substrate in the presence and absence of glucose aerobically and anaerobically. Almost no radioactivity could be found in either the produced $CO_2$ or any

1003540159

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

Information concerning the activity of the α-glycerophosphate shuttle (6). Lungs will also be perfused with iodoacetamide to inhibit the activity of glyceraldehyde-3-phosphate dehydrogenase. Tissue levels of α-glycerophosphate and dihydroxyacetone phosphate will be measured to determine α-glycerophosphate shuttle activity. These experiments with inhibitors will also be done with glycerol and xylitol as substrates to determine if α-glycerophosphate can be produced in lung tissue via glycerol kinase and via the hexomonophosphate shunt. Because α-glycerophosphate is thought to be a key intermediate in phospholipid production (7), knowledge of the potential metabolic sources of this compound is important. If the glycerophosphate shuttle appears to be absent, an evaluation will be attempted of the malate-aspartate shuttle.

3. Measurement of glucose uptake aerobically and anaerobically.

For the measurement of glucose uptake, rat lung slices are incubated with $^{14}C$ uniformly labeled glucose under the appropriate condition in the Warburg apparatus. At suitable time intervals the reaction is quenched with 7% perchloric acid, and the change in glucose concentration is measured either by enzymatic techniques (8-10) or by the method used in I. A. Rose's laboratory using column chromatography. The neutralized solution is put on a Dowex-1 acetate column with some carrier glucose, lactate, etc. Because the glucose is uncharged, it will pass right through, while the Krebs cycle acids, phosphate esters, etc., will stick. The glucose therefore elutes in the region of the breakthrough peak. The acidic components are then eluted using an HCl gradient, and by the use of cold carriers the fraction can be analyzed and counted. By this method we hope to be able to quantitate the distribution of the $^{14}C$ and measure the glucose uptake. Lactic acid production will be measured by standard enzymatic techniques (11), and oxygen utilization and $CO_2$ production will be measured by standard manometric techniques (1).

4. Determination of the rate of production or utilization and the origin of lactic acid aerobically and anaerobically.

It is important to measure the rate as well as the origin of lactic acid production anaerobically and aerobically in the presence and absence of a substrate. For substrate we intend to use initially glucose, a carbohydrate, and a 4% rat serum albumin containing bound palmitate or octanoic acid as a representative lipid. Oxygen utilization, $CO_2$ production, and lactic acid production will also be determined. Comparison of the $C^{-1}/C^{-6}$ ratio of $CO_2$ production both anaerobically and aerobically, utilizing C-1 and C-6 labeled glucose permits an estimate to be made of the contribution of the pentose shunt. $CO_2$ radioactivity will be measured along with oxygen utilization and lactic acid production.

5. Determination of the precursor origin of phospholipid.

We have shown that lactic acid is produced in significant amounts. Our initial experiments indicate that the lung can utilize none of this lactate as a substrate, but this must be confirmed by further investigation. Utilizing uniformly labeled $^{14}C$-acetate as the substrate both anaerobically and aerobically, measurements of radioactive $CO_2$, oxygen consumption and fractionation into acid soluble fractions, lipids, proteins, RNA, DNA, etc., will be performed (12). Subsequent determination of radioactivity will indicate the distribution of substrate byproducts. It is possible that the glucose and the palmitate moieties

100354164

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

accounted for by $CO_2$, 25% in protein, 2.5% in RNA, and 4.3% in lipids. More data must be obtained to confirm these preliminary results before abnormal situations can be effectively investigated.

References:


1. Felts, J.M. Health Phys. 10:973, 1964.
2. Felts, J.M. Med. Thorac. 22:89, 1965.
3. Geyer, R.P., Matthews, L.W., and Stare, F.J. J. Biol. Chem. 180:1037, 1949.
4. Reiss, O.K. In, Current Research in Chronic Airways Obstruction: Ninth Aspen Emphysema Conference. D.L. Kelble, W.G. Rainer, and G.F. Filley, eds. U.S. Government Printing Office, Washington, D.C., 1968, pp. 35-42.
5. Harlan, W.R., Jr., Said, S.I., Spiers, C.L., Banerjee, C.M., and Avery, M.E. Clin. Res. 12:291, 1964.
6. Nasr, R., and Heinermann, H.D. Amer. J. Physiol. 208:118, 1965.
7. Theodore, J., Robin, E.D., and Gee, J.B.L. In, Current Research in Chronic Airways Obstruction: Ninth Aspen Emphysema Conference. D.L. Kelbe, W.G. Rainer, and G.F. Filley, eds. U.S. Government Printing Office, Washington, D.C., 1968, pp. 3-11.
8. Sorokin, S.P. Develop. Biol. 3:60, 1961.
9. Holub, M. Folia Microbiol. 14:359, 1969.
10. Gee, J.B.L., Vassallo, C.L., Bell, P., Kaskin, J., Basford, R.E., and Field, J.B. J. Clin. Invest. 49:1280, 1970.
11. Hibbs, J.B., Jr., Lambert, L.H., Jr., and Remington, J.S. Science 177:998, 1972.
12. Sorokin, S.P. In, Morphology of Experimental Respiratory Carcinogenesis. P. Nettesheim, M.G. Hanna, Jr., and J.W. Deatherage, Jr., eds. U.S. Atomic Energy Commission, Div. of Technical Information, Oak Ridge, Tenn., 1970, pp. 3-43.
13. Pinkett, M.O., Cowdrey, C.R., and Nowell, P.C. Amer. J. Clin. Path. 48:859, 1966.
14. Massaro, D., Simon, M.R., and Steinkamp, H. J. Appl. Physiol. 30:1, 1971.
15. Weber, K.C., and Visscher, M.B. Amer. J. Physiol. 217:1044, 1969.
16. Glaviano, V.V., Yo, S., and Masters, T. Amer. J. Physiol. 213:437, 1967.
17. Bucherl, R., Bucherl, E.S., Heimburg, P., and Schwab, M. Klin. Wschr. 36:1078, 1958.
18. Lochner, W., and Nasseri, M. Pflugers Archiv. 272:180, 1960.
19. Salisbury-Murphy, S., Rubinstein, D., and Beck, J.C. Amer. J. Physiol. 211:988, 1966.
20. Wolfe, B.M.J., Rubinstein, D., and Beck, J.C. Canad. J. Biochem. 46:151, 1968.
21. Tyler, W.S., and Pearse, A.G.E. Thorax 20:149, 1965.
22. Fisher, A.B., Scarpa, A., LaNoue, K., and Williamson, J.R. Physiologist 14:142, 1971.
23. Mitchell, A.M., and Cournand, A. J. Clin. Invest. 34:471, 1955.
24. Okamoto, Y. Biochem. Z. 160:52, 1925.
25. Warburg, O., et al. Arch. Biochem. Biophys. 78:573, 1958.
26. Lehninger, A.L. J. Biol. Chem. 190, 1951.
27. Borst, P. Biochim. Biophys. Acta 57:270.
28. Boxer, G.E., and Devlin, T.M. Science 134:1495, 1961.
29. Milstein, S.W., and Driscoll, L.H. J. Biol. Chem. 235:19, 1959.
30. Weinhouse, S. Science 124:267, 1956.


10035162

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

Recent studies of lung mitochondria by Fisher et al. (personal communication; 22) suggest that little oxidative phosphorylation occurs. They feel that the moderate amount of ATP produced is utilized during phospholipid synthesis under the control of calcium ion.

All of the above data support our contention that the regulation of metabolic processes of lung tissue is unusual. Of interest and probable importance is the question, why does the organ with the greatest access to oxygen have to depend primarily upon anaerobic mechanisms and produce lactate (which it apparently is incapable of metabolizing efficiently). There are conflicting experiments with perfused lung (23), which, in contradistinction, showed no change or utilization of lactate in blood during passes through the lungs. Thus it appears that factors other than oxygen or substrate availability might control the oxidative metabolism of the lung.

It is of extreme interest, and perhaps of some importance, that many malignant tumors also have a high glycolytic capacity, as first described by Warburg. It is evident that the glycolysis is an important source of energy in cancer tissue, sufficient even for prolonged survival in the absence of oxygen. Thus, transplantable tumor may grow again after having been kept for three days in the absence of oxygen (24,25). This means that neoplastic tissues share with other tissues the capacity to obtain energy from two sources. The proportion of energy derived from glycolysis is very high in neoplastic tissue. The reasons for the excessive lactate production of neoplastic cells have only recently been elucidated. It is clear that extramitochondrial reduced pyridine nucleotides are not directly oxidized by the mitochondrial respiratory chain of animal cells. Lehninger (26) first observed that intact mitochondria isolated from liver were incapable of oxidizing added DPNH. Similar observations have been made with mitochondria of ascites tumor cells (27,28). The slow rate of penetration of pyridine nucleotides into liver mitochondria in the intact animal (12,29) confirms the conclusion drawn from the in vitro studies. It is now clear that an indirect mechanism by a shuttle system is utilized for the introduction of extra mitochondrial reducing equivalents to the mitochondrial respiratory chain. The high aerobic glycolysis in tumor cells has been ascribed to the lack of such a shuttle system (28,30,31). Among these systems, the α-glycerophosphate shuttle has been extensively studied, and a low activity of the cytosolic enzyme involved in this shuttle, the α-glycerophosphate dehydrogenase, has been considered typical of tumors with high rates of aerobic glycolysis (32). Such a defect may also be present in normal lung tissue, and, if so, would make this tissue exciting to study and compare with biochemical mechanisms occurring with neoplastic alterations. There are, of course, many other biochemical characteristics which distinguish neoplastic tissue from other tissue (33). Together, they are responsible for uncontrolled growth, but from a quantitative point of view, no single characteristic is as striking as the aerobic glycolysis.

2. Work accomplished by the applicants.

a. Lactate production of lung.

In 1958 Bucherl et al. (17) studied the A-V difference of lactic acid in 27 patients during cardiac catheterization and found a significant increase in lactic acid in the left atrial blood compared with the pulmonary artery blood. We repeated this study and obtained similar

10035401158

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

We will also use the isolated, perfused lung to study pathways of free fatty acid metabolism. As noted previously, there is considerable discrepancy in the literature about the ability of lung to oxidize fatty acids. $^{14}C$-labeled palmitate and octanoate bound to albumin (19) will be added to the perfusion mixture, and the $^{14}CO_2$ evolved will be measured. The rate of change of free fatty acids in the perfusate will be measured spectrophotometrically (20). Further studies will depend on these initial results. For example, if palmitate oxidation is impaired, a palmitylcarnitine transferase inhibitor, (+) decanoylcarnitine, will be added to test for the requirement of carnitine for palmitate oxidation (21), and carnitine tissue levels will be measured.

We will further study oxidative metabolism of the lung by applying the technique of surface fluorometry, using a spinning disc fluorometer (22). For these experiments, the lungs will be insufflated with air or oxygen, and ventilation will be suspended. The fluorescence probe will be applied to the lung surface while perfusion continues. We will follow the fluorescence changes as the perfusion medium is altered with respect to substrate concentration and composition, and this will be compared to fluorescence changes produced by ventilating the lung with $N_2$. With this technique we can simultaneously monitor fluorescence changes due to pyridine nucleotides and, with an absorption channel, measure changes due to flavoproteins. In this way we can pick up the respiratory chain at several sites to determine the effect of substrate in a physiological setting on intracellular redox state.

9. Comparison of animal data with human data.

After we have acquired an adequate amount of information about the pathways and regulation of metabolism from our animal studies, we will carry out comparative investigations of normal human pulmonary tissue. Tissues will be obtained from operative specimens taken from patients undergoing surgical procedures for known medical indications.

10. Individual cell components.

At future stages in this project, such cells of the pulmonary parenchyma as can be isolated by standard techniques, such as alveolar macrophages, will be individually investigated for their role in the overall metabolic processes, which we will understand better at that time.

1003540167

11. Biosynthetic pathways.

In future stages of the program, studies of biosynthetic pathways and their regulation will be carried out for surface-active substances and vasoactive substances (including prostaglandin) which appear to be either synthesized or degradated by the lung. Techniques described above with both isolated, perfused lung and lung slices will be utilized. Confirmatory in vivo experiments will be carried out when feasible.

12. Evaluation of the effects of smoke upon pulmonary metabolism.

Utilizing techniques similar to those described above, the effects upon normal pulmonary metabolism of tobacco smoke will be determined. In one group of experiments in vitro evaluation will be determined in closed vessels containing

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

either smoke or some of its components. In in vivo experiments animals will be placed in plexiglass chambers in which the atmospheric conditions of $O_2$ and $CO_2$ and the percent of smoke can be controlled. The exposure time will vary from short-term (a few hours or days) to long-term (one to four weeks). Physiologic measurements of pulmonary function will be carried out where feasible. At the end of this period the animals will be sacrificed, and either lung slices or perfusion techniques will be utilized for the evaluation of metabolism. In particular, we plan to investigate the Ca++ and Mn++ transport of mitochondria. These ions have been shown to be affected by local anesthetics (23). It is to be noted that in this investigation no attempt will be made to design new methods of instrumentation or technology. We will use well-established experimental techniques in an effort to gain new information and insights into pulmonary metabolism.

References:


1. Umbreit, W.W., Burris, R.H., and Stauffer, J.S. Manometric Technique. Burgess, Minneapolis, 1964.
2. Kleinzeller, A. Manometrische Methoden. Verlag, Prague, 1964.
3. Henry, R.J. Clinical Chemistry: Principles and Technics. Hoeber Medical Division, Harper and Row, New York, 1967.
4. Williamson, J.R., and Corkey, B.E. In, Methods in Enzymology, Vol. 13, J.M. Lowenstein, ed. Academic Press, New York, 1969, p. 434.
5. Gould, K.G., Jr., et al. Science 178:1209, 1972.
6. Safer, B., Smith, C.M., and Williamson, J.R. J. Mol. Cell. Cardiol. 2:111, 1971.
7. Felts, J.M. Health Phys. 10:973, 1964.
8. Schmidt, F.H. Klin. Wochschr. 39:1244, 1961.
9. Hill, J.D., and Kessler, E. J. Lab. Clin. Med. 57:970, 1961.
10. Bergmeyer, H.U. Methods of Enzymatic Analysis. Academic Press, New York, 1964.
11. Boehrmger and Sons, Analytical Kits.
12. Fleck, A., and Munro, H.N. Biochim. Biophys. Acta 55:571, 1962.
13. Said, S., Klein, R.M., Norrell, L.W., and Maddox, Y.T. Science 152:657, 1966.
14. Felts, J.M. Med. Thorac. 22:89, 1965.
15. Atkinson, D.E. Ann. Rev. Biochem. 35:85, 1966.
16. Mela, L., and Chance, B. Biochemistry 7:4059, 1968.
17. O'Neill, J.J., and Tierney, D.F. Fed. Proc. 30:697, 1971.
18. Veech, R.L., Eggleston, L.V., and Krebs, H.A. Biochem. J. 115:609, 1969.
19. Milstein, S.W., and Driscoll, L.H. J. Biol. Chem. 234:19, 1959.
20. Novak, M. J. Lipid Res. 6:431, 1965.
21. Nasr, K., and Heinmann, H.O. Amer. J. Physiol. 208:118, 1965.
22. Scholz, R., et al. J. Biol. Chem. 244:2317, 1969.
23. Mela, L. Arch. Biochem. Biophys. 123:286, 1968.


10035401G8

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000

31. Gordon, E.E., Ernster, L., and Dallner, G. Cancer Res. 27:1372, 1967.
32. Knox, W.E. In, Advances in Cancer Research, A. Haddow and S. Weinhouse, eds., Vol. II, Academic Press, New York, 1967, p. 117.
33. Weber, G. Gann-Monograph No. 13, Japanese Cancer Association, 1972.

B. Methods of procedure.

The experiments in the early part of the investigation will be done first with rat lungs and then with dog lungs. The basic experiments, as we now conceive them, are described below. The experimental design and direction will depend, however, on the results of preceding experiments.

1. Confirmation of preliminary data and investigation of substrate utilization.

Initially, we intend to elucidate and confirm our early findings (see "Background") by means of the standard Warburg manometric technique (1,2). Rat lung slices are cut free-hand by standard techniques and placed in 5-ml Warburg vessels with buffered physiologic solution at pH 7.4 and an osmolarity of 280 to 300 at 37°C. The center well contains 0.7 ml of 20% potassium hydroxide for $CO_2$ trapping with the filter paper in place to increase the area of absorption of $CO_2$. Various radioactive and nonradioactive substrates are added (see below), and incubations are carried out for suitable time periods. Currently, we are using 30, 60, 90, and 120 minutes. The reaction is stopped with perchloric acid, and the indicated analyses are performed. The data are expressed per milligram of nitrogen or wet weight. Nitrogen analysis will be performed by the standard micro Kjeldahl techniques (3). Thd necessary control experiments will be run concurrently with liver. All anaerobic experiments will be carried out with 100% nitrogen.

2. Evaluation of the glycerophosphate shuttle.

Because of the depressed activity of the α-glycerophosphate enzyme in the cytosol of neoplastic tissue, which impairs the shuttle system of cancer cells, we feel that an initial approach would be to see if the same sort of mechanism was present in normal pulmonary tissue. Utilizing standard techniques, the cytosol of lung tissue will be collected and the α-glycerophosphate dehydrogenase activity will be measured fluorometrically (4). Utilizing tissue slices, and later isolated lung cells prepared by the method of Gould (5), the tissue levels of α-glycerophosphate and dihydroxyacetone will be evaluated utilizing fluorometric techniques (4). Although we are aware of the problems involved and the objections to the use of tissue homogenates, perhaps some of these experiments, for technical reasons, will be performed with this type of preparation.

The significance of the α-glycerophosphate pathway as an NADH shuttle mechanism between cytoplasmic and mitochondrial spaces in lung cells will be studied by experimentally manipulating the tissue level of α-glycerophosphate with the use of inhibitors. First, amino-oxyacetate will be added to the perfusion period in order to inhibit the flux of reducing equivalents via the malate-aspartate shuttle mechanisms (6). Lactate or glucose will be used as substrate, and aliquots of the perfusion fluid and freeze-clamped tissue will be analyzed for metabolic intermediates by fluorometric methods (4). Analysis of the effect of the inhibition on the rate of aerobic glycolysis and lactate oxidation will give

10035540163

Source: https://www.industrydocuments.ucsf.edu/docs/jlll0000